Generation and Screening of a Non-typeable *Haemophilus influenzae* Tn-seq Mutant Library

Jeroen D. Langereis*

Laboratory of Pediatric Infectious Diseases, Department of Pediatrics and Laboratory of Medical Immunology, Department of Laboratory Medicine, Radboud University Medical Centre, Nijmegen, The Netherlands

*For correspondence: Jeroen.Langereis@radboudumc.nl

[Abstract] The genome-wide screen Tn-seq (van Opijnen *et al.*, 2009) is very valuable tools to identify bacterial genes with a conditionally essential function, for instance genes involved in bacterial virulence. These techniques are based on the generation of a random mutant library, which is grown in a control of challenge situation (Figure 1). The advantage of using a mariner transposon for the generation of a random transposon mutant library is its insertion into TA sites, which makes the insertion in the genome highly random. In addition, an MmeI restriction site can be introduced in the inverted repeat of the transposon, without affecting the recognition by HimarC9 transposase.

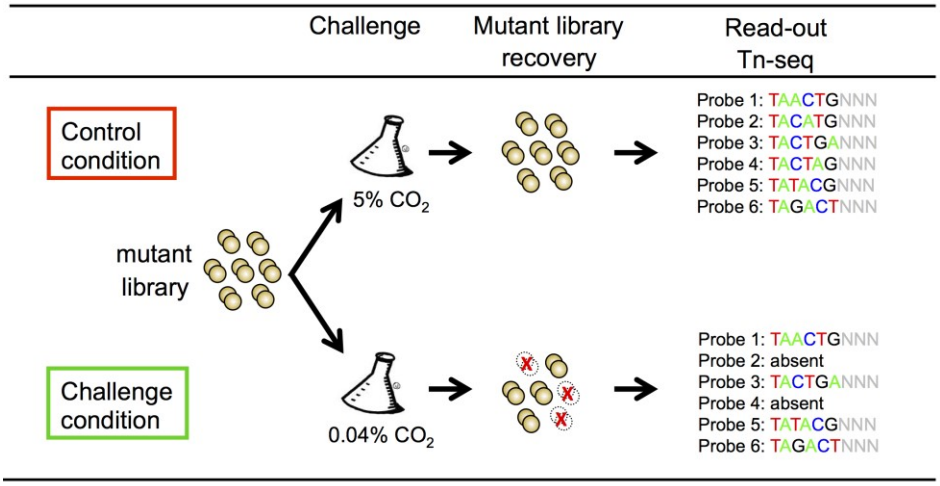

Figure 1. Schematic representation of the non-typeable *Haemophilus influenzae* tn-seq mutant library screen for survival and growth in environmental air

Materials and Reagents

1. 1 U/µl Calf Intestinal Alkaline Phoshatase (CIAP) (New England Biolabs, catalog number: M0290S)
2. Chloroform: isoamyl alcohol
3. Phenol: chloroform: isoamayl alcohol
4. Milli-Q water

5. 10 mM dNTP mix (New England Biolabs, catalog number: N0447S)
6. 1 mM dNTP mix
7. Absolute ethanol
8. 70 % Ethanol
9. 10 mg/ml Glycogen
10. 2 U/µl MmeI restriction enzyme (New England Biolabs, catalog number: R0637S)
11. 10x NEBuffer 4
12. 32 mM S-denosylmethionine
13. 3 M NaAc (pH 5.3)
14. 5 M NaCl
15. 2 U/µl Phusion DNA polymerase (New England Biolabs, catalog number: M0530S)
16. 5x Phusion HF buffer
17. 10 U/µl T4 DNA ligase (New England Biolabs, catalog number: M0202S)
18. 20 U/µl T4 DNA ligase
19. 10x T4 DNA ligase buffer
20. 2.5 U/l T4 DNA polymerase (New England Biolabs, catalog number: M0203S)
21. 10x T4 DNA polymerase buffer
22. T4 polynucleotide kinase (3' phosphatase minus) (New England Biolabs, catalog number: M0236S)
23. 100x TE buffer
24. 1 M NaOH,
25. 50 % Glycerol
26. 1 mM DTT
27. 5 M NaCl
28. 1 M $MgCl_2$
29. 10 mg/ml BSA
30. 5 U/ml *E.coli* DNA ligase (New England Biolabs, catalog number: M0205S)
31. 10x *E.coli* DNA ligase buffer
32. 1 M Hepes (pH 7.9)
33. HimarC9 transposase
34. M-IV medium (Herriott *et al.*, 1970)
35. 1 mg/ml Hemin (Sigma-Aldrich, catalog number: H9039)
36. 10 mg/ml Nicotinamide adenine dinucleotide (NAD) (Sigma-Aldrich, catalog number: N7004)
37. Brain heart infusion medium (BHI) (BD Biosciences, catalog number: 237500)
38. Supplemented BHI, BHI medium containing 10 µg/ml Hemin and 2 µg/ml NAD
39. Bacto-agar (BD Biosciences, catalog number: 212030)
40. Supplemented BHI plates, sBHI containing 1.5% bacto agar
41. PBS
42. 100 mg/ml RNase A (Roche Diagnostics, catalog number: 10109142001)

43. Qiagen Genomic-tip 20/G columns (QIAGEN, catalog number: 10223)
44. Qiagen Genomic DNA buffer set (QIAGEN, catalog number: 19060)
45. Minelute Reaction Cleanup Kit (QIAGEN, catalog number: 28204)
46. Qubit dsDNA BR assay (Life technologies, catalog number: Q32850)
47. Acceptor DNA

    Any type of DNA can serve as acceptor for in vitro mariner transposition. The most common types of acceptor DNA are: Chromosomal DNA of the target strain (High quality DNA is required, preferably isolated with Qiagen Genomic Tip columns) or PCR products of target genes or regions.
48. Donor DNA

    Any type of DNA that carries a mariner transposon with MmeI restriction site in the inverted repeat can serve as donor for transposon in the in vitro mariner transposition reaction. Used pGSF8 plasmid, carrying transposon with spectinomycin resistance cassette, suitable for GAF and TnSeq (Langereis *et al.*, 2013).
49. Primers used for sequence adapters ligation and PCR amplification (see Appendixes)

Equipment

1. Pipet tips: 0.5-10 l, 2-20 l, 20-200 l 100-1000 l
2. 15 cm dishes
3. Heating block for incubations ranging from 16 °C and 75 °C (Grant QBD2)
4. Microcentrifuge for 1.5 ml tubes (Eppendorf, model: 5417R)
5. Centrifuge for 50 ml tubes (Eppendorf, model: 5810)
6. T100 thermal cycler (Bio-Rad Laboratories)
7. Nanodrop spectrophotometer (Thermo Fisher Scientific, Nanodrop, model: ND1000)
8. Incubator with 5% CO_2 (BINDER GmbH, model: CB 150)
9. Qubit Fluorometer (Life Technologies)
10. Bioanalyser (Agilent Technologies)

Procedure

This detailed protocol is divided into four sections:

Part I. Generation of mutant library

We provide a detailed protocol for the generation of a mutant library in non-typeable *Haemophilus influenzae*, but this can be used for all bacteria that are naturally competent.

Part II. Mutant library screen

As example, we provide a detailed protocol for the identification of non-typeable *Haemophilus* genes essential for survival in environmental air, as published before (Langereis *et al.*, 2013).

Part III. Mutant library readout

Part IV. Data analysis

For data analysis, the web-based analysis software ESSENTIALS was used (Zomer *et al.*, 2012). A detailed manual can be found on the website (http://bamics2.cmbi.ru.nl/websoftware/essentials/essentials_start.php).

Part I. Generation of mutant library

A. Transposition reaction

1. Prepare the 6x transposition buffer enough for 30 reactions fresh by combining
   60 µl 50 % glycerol
   0.6 µl 1 M DTT
   7.5 µl Hepes (pH 7.9)
   7.5 µl 10 mg/ml BSA
   6.0 µl 5 M NaCl
   3.0 µl 1 M $MgCl_2$
   15.4 µl sterile dH_2O
2. Combine in a 1.5 ml tube
   3.3 µl 6x Transposition Buffer
   0.5-1.0 µg recipient DNA
   0.5-1.0 µg donor for mariner transposon
   1 µl recombinant Himar1 transposase
   Sterile dH_2O until V_{total}= 20 µl
   Mix and incubate for about 4 h at 30 °C in a heating block
3. Inactivate transposase for 10 min at 75 °C in a heating block
4. Add to inactivated transposition reaction:
   2 µl 3 M Sodium Acetate (pH ~5.3)
   0.5 µl 20 mg/ml glycogen
   50 µl 100% ethanol
   Mix and place in -20 °C freezer for at least 30 min
5. Centrifuge at maximum speed in a precooled (4 °C) microcentrifuge for 15 min.
6. Remove supernatant with 1 ml pipet (do not touch the pellet).
   Note: At this moment it is not necessary to carefully remove al liquid.
7. Add 250 µl of 70% ethanol (just add, do not try to resuspend the pellet).
8. Centrifuge at maximum speed in a precooled (4 °C) microcentrifuge for 5 min.
9. Carefully remove all supernatant with a 1 ml pipet (do not touch the pellet).
   (Optional: Centrifuge in a microcentrifuge for a few seconds to collect the remainder of the supernatant at the bottom of the tube and carefully remove all supernatant with a 200 µl pipet.)
10. Dry the pellet on air (pellets turns from opaque to white in ~30 min).
    (Optional: Place the tubes in a heating block at 30 °C to speed up evaporation of liquid.)

11. Dissolve pellet in 15.8 µl sterile dH_2O

B. Repair of the transposition reaction

12. Add to the dissolved pellet
    2 µl 10x T4 DNA polymerase Reaction Buffer
    0.2 µl 10 mg/ml BSA
    1 µl 1 mM dNTP mix
    1 µl 2.5 U/l T4 DNA polymerase
    Incubate for 30 min at 16 °C
13. Inactivate polymerase for 10 min at 75 °C in a heating block.
14. Add to inactivated polymerase reaction
    2 µl 3 M Sodium Acetate
    0.5 µl 20 mg/ml glycogen
    50 µl 100% ethanol
    Mix and incubate in -20 °C freezer for at least 30 min.
15. Centrifuge at maximum speed in a precooled (4 °C) microcentrifuge for 15 min.
16. Remove supernatant with 1 ml pipet (do not touch the pellet).
    Note: at this moment it is not necessary to carefully remove al liquid.
17. Add 250 µl of 70% ethanol.
18. Centrifuge at maximum speed in a precooled (4 °C) microcentrifuge for 5 min.
19. Carefully remove all supernatant with a 1 ml pipet (do not touch the pellet).
    (Optional: Centrifuge in a microcentrifuge for a few seconds to collect the remainder of the supernatant at the bottom of the tube and carefully remove all supernatant with a 200 µl pipet.)
20. Dry the pellet on air (pellets turns from opaque to bright white in ~30 min).
    (Optional: place the tubes in a heating block at 30 °C to speed up evaporation of liquid.)
21. Dissolve pellet in 17.8 µl sterile dH_2O.
22. Add to the dissolved pellet:
    2 µl 10x *E.coli* DNA ligase Reaction Buffer
    0.2 µl *E.coli* DNA ligase (5 U/µl)
    Incubate overnight at 16 °C.
23. Store mutagenized DNA at -20 °C.

C. Transformation

24. Grow 10 ml non-typeable *Haemophilus* influenzae (NTHi) in BHI medium containing 10 µg/ml hemin and 2 µg/ml NAD shaking with 225 rpm at 37 °C to an OD_{620} of 0.3.
25. Centrifuge the bacteria 10 min with 3,000 *x g* and resuspend in 10 ml PBS.
26. Centrifuge the bacteria 10 min with 3000 *x g* and resuspend in 10 ml M-IV medium and incubate 100 min shaking with 100 rpm at 37 °C.
27. Centrifuge the bacteria 10 min with 3000 *x g* and resuspend in 1 ml M-IV medium and transfer to a 1.5 ml tube.

28. Add 1-5 µg mutagenized DNA and incubate 60 min with 100 rpm at 37 °C.
29. Plate 1 to 100 µl per sBHI plate for over night growth at 37 °C + 5% CO_2.
30. Collect the colonies by adding 5 ml PBS + 15% glycerol on the plates and store 1 ml aliquots at -80 °C.

    Note: The number of colonies (transposon mutants) is dependent on the transformation efficiency of the NTHi strain used in this experiment. It is recommended to use a highly competent NTHi strain in order to obtain large mutant libraries. Alternatively, multiple transformations can be pooled to obtain sufficient transposon mutants, typically 10-20-fold the number of genes in the genome.

Part II. Mutant library screen

The mutant library can be used in any control and stress condition. As proof of principle, we have used growth in air enriched with 5% CO_2 (control condition) or ambient air with 0.04% CO_2 (stress condition). To do so, the mutant library was constructed with 5% CO_2 enriched M-IV medium (M-IV medium incubated at least 2 hours at 5% CO_2 in an open 50 ml tube) without shaking to prevent loss of mutants while making the mutant library (generation mutant library steps 27-28).

1. Thaw 1 ml aliquot of the NTHi mutant library at RT.
2. Centrifuge 2 min at 10,000 *x g* in microcentrifuge.
3. Remove the medium containing glycerol and resuspend the bacteria in 1 ml sBHI medium.
4. Grow the mutant library in 5 ml sBHI medium enriched with 5% CO_2 (incubate BHI medium overnight in the incubator + 5% CO_2 and add hemin and NAD fresh before use) without shaking to OD_{620} = 0.5 and store three 1 ml aliquots with 15% glycerol at -80 °C. (Start culture)
5. Dilute the start culture 1:100 in 5 ml 5% enriched sBHI medium (control) or sBHI medium (stress) and grow to OD_{620} = 0.5 with 5% CO_2 (control) or ambient air (stress) at 37 °C and store three 1 ml aliquots with 15% glycerol at -80 °C for storage and start culture for next round. (Round 1)
6. Dilute the first round culture 1:100 in 5 ml 5% enriched sBHI medium (control) or sBHI medium (stress) and grow to OD_{620} = 0.5 with 5% CO_2 (control) or ambient air (stress) at 37 °C and store three 1 ml aliquots with 15% glycerol at -80 °C for storage and start culture for next round. (Round 2)
7. Dilute the second round culture 1:100 in 5 ml 5% enriched sBHI medium (control) or sBHI medium (stress) and grow to OD_{620} = 0.5 with 5% CO_2 (control) or ambient air (stress) at 37 °C and store three 1 ml aliquots with 15% glycerol at -80 °C for storage. (Round 3)
8. Thaw the challenged mutant library at RT, centrifuge 3 min 10.000 *x g* and resuspend the bacteria in 1 ml buffer B1 (Qiagen) supplemented with 2 µl RNase A solution (100 mg/ml).

Part III. Mutant library readout

A. Mutant library chromosomal DNA isolation and digestion

1. Thaw the challenged mutant library at RT, centrifuge 3 min 10,000 *x g* and resuspend the bacteria in 1 ml buffer B1 supplemented with 2 µl RNase A solution (100 mg/ml).
2. Isolate the chromosomal DNA from the challenged mutant libraries with Qiagen Genomic Tip columns.
   a. Add 20 µl lysozyme (100 mg/ml) and 45 µl proteinase K (10 mg/ml) and incubate 30 min at 37 °C.
   b. Add 350 µl buffer B2 and incubate 30 min at 50 °C.
   c. Place a Qiagen genomic tip 20/G column on a 15 ml tube and the column with 2 ml buffer QBT.
   d. Vortex the sample and apply it to the equilibrated column.
   e. Wash the column 3x with 1 ml buffer QC.
   f. Replace the 15 ml tube and elute the DNA with 2x 1 ml buffer QF.
   g. Transfer 3x 650 µl buffer to a 1.5 ml tube, add 455 µl RT isopropanol and centrifuge immediately 15 min. with max. speed at 4 °C.
   h. Remove the isopropanol and wash with 1 ml cold 70% ethanol. Vortex briefly and centrifuge 10 min. with max. speed at 4 °C.
   i. Remove the ethanol and wash a second time with 1 ml cold 70% ethanol. Vortex briefly and centrifuge 10 min. with max. speed at 4 °C.
   j. Remove the ethanol and let the pellet dry. Do not completely dry the pellet.
   k. Resuspend the DNA pellet in 100 µl TE.
      (Optional: Incubate at 50 °C to dissolve the DNA pellet.)
3. Prepare reaction mixture in 1.5 ml microfuge tube
   2 µg chromosomal DNA of mutant library
   5 µl 2 U/µl MmeI (=10 U)
   20 µl 10x NEBuffer 4
   0.3 µl 32 mM S-adenosylmethionine
   V_{total} with dH_2O= 200 µl
   Incubate at 37 °C, >4 h
4. Add 1 µl 1U/µl CIAP and mix (=1 U) and incubate at 50°C, 30 min.
5. Add 200 µl phenol:chloroform:isoamayl alcohol and vortex 10 sec.
6. Centrifuge max speed, 5 min at RT.
7. Transfer upper layer (~200 µl) to new 1.5 ml microfuge tube with 200 µl chloroform: isoamyl alcohol and vortex 10 sec.
8. Centrifuge max speed, 5 min at RT.
9. Transfer upper layer (~200 µl) to new 1.5 ml microfuge tube.
10. Add to the tube:
    20 µl 3 M Sodium Acetate
    0.5 µl 20 mg/ml glycogen

500 µl 100% ethanol

Mix and incubate in -20 °C freezer for at least 30 min.

11. Centrifuge at maximum speed in a precooled (4 °C) microcentrifuge for 15 min.
12. Remove supernatant with 1 ml pipet (do not touch the pellet).

    Note: At this moment it is not necessary to carefully remove al liquid.
13. Add 500 µl of 70% ethanol.
14. Centrifuge at maximum speed in a precooled (4 °C) microcentrifuge for 5 min.
15. Carefully remove all supernatant with a 1 ml pipet (do not touch the pellet).

    (Optional: Centrifuge in a microcentrifuge for a few seconds to collect the remainder of the supernatant at the bottom of the tube and carefully remove all supernatant with a 200 µl pipet.)
16. Dry the pellet on air (pellets turns from opaque to bright white in ~30 min).

    (Optional: Place the tubes in a heating block at 30 °C to speed up evaporation of liquid.)
17. Dissolve pellet in 20 µl dH_2O
18. Measure the DNA concentration

B. Adapter annealing

For each adapter, a F- and R-primer must be annealed (see primers listed in the appendix). A total of 12 adapters are listed in the appendix, but the number needed for the experiment (*e.g.* 4 or 8) can be annealed in parallel in separate tubes.

19. For primer annealing, prepare mix in 1.5 ml microfuge tube:

    5 µl 1 nmol/µl F primer

    5 µl 1 nmol/µl R primer

    0.5 µl 100x TE

    0.5 µl 5 M NaCl

    39 µl dH_2O

    Incubate for 10 min at 95 °C in heating block
20. Remove metal tube holder from heating block and allow to cool slowly on bench to T< 30 °C.
21. Store samples at -20 °C until further use (annealed adapters can be stored > 1 year).
22. For 5'-phosphorylation of the annealed adapters, prepare mix in 1.5 ml microfuge tube:

    2 µl annealed adapter (100 pmol/µl)

    2 µl 10x T4 DNA ligase buffer

    0.5 µl 10 U/µl T4 polynucleotide kinase (3' phosphatase minus)

    15.5 µl dH_2O
23. Incubate for 5 min at 37 °C.
24. Incubate 10 min at 70 °C in a heat block.

25. Remove metal tube holder from heating block and allow to cool slowly on bench to T< 30 °C.

C. Adapter ligation and PCR amplification

26. MmeI restriction fragments and annealed adapters were ligated in the following reaction mixture:
    100 ng dephosphorylated MmeI restriction fragments
    0.2 µl (10 pmol/µl) freshly phosphorylated annealed adapter
    2 µl 10x T4 DNA ligase buffer
    0.2 µl (10 U/µl) T4 DNA ligase
    V_{total} with dH_2O= 20 µl
    Incubate for over night at 16 °C
27. Ligated adapter and restriction fragments were PCR amplified in the following reaction mixture:
    26 µl dH_2O
    10 µl 5x Phusion HF buffer
    1 µl 10 mM dNTP mix
    5 µl (4 pmol/µl) PBGSF23 primer
    5 µl (4 pmol/µl) PBGSF31 primer
    2.5 µl ligation mixture
    0.5 µl Phusion DNA polymerase
    Incubate reaction according to the following PCR program
    72 °C - 1 min
    98 °C - 30 sec
    98 °C - 10 sec |
    57 °C - 30 sec | 25x
    72 °C - 10 sec |
    72 °C - 5 min
28. Check 2 µl of the PCR reaction on a 2.5 % agarose gel with a 100 bp ladder. (PCR product should be a single band of ~125 bp.)
29. Cleanup PCR reaction with Minelute Reaction Cleanup Kit
    a. Mix 50 µl PCR reaction with 50 µl dH_2O
    b. Add 300 µl ERC buffer
    c. Apply to MinElute column in 2 ml tube; centrifuge 1 min at 18,000 rpm.
    d. Discard flow-through, reuse 2 ml tube.
    e. Add 750 µl buffer PE; centrifuge 1 min at 18,000 rpm.
    f. Discard flow-through, reuse 2 ml tube.
    g. Centrifuge 1 min at maximum speed to completely dry membrane.
    h. Place MinElute column in RNase-free 1.5 ml tube.
    i. Pipet 10 µl dH_2O on center of the column membrane; wait 1 min.

j. Centrifuge 1 min at maximum speed.

30. Measure DNA concentration with Nanodrop using d H_2O as blank.
31. Combine equimolar amounts of differently barcoded DNA probes in one tube.
32. For quality control, perform a Qubit DNA concentration measurement and a bioanalyser run.
33. 9 fmol of DNA probe was loaded on a Genome Analyzer II (Illumina) for sequence analysis according to the manufacturer's protocols, using a Genomic DNA Sequencing Primer (Illumina) and 36 sequencing cycles.
    For further information about Illumina sequencing see Zomer *et al.* (2012).

Part. IV. Data analysis

Data analysis is in detail described in Langereis *et al*. (2013).

1. Generate FASTQ files with 35 bp sequences.
   Note: The first nucleotide of the Genome Analyzer II (Illumina) 36-bp sequence reads often has a poor quality and is therefore omitted.
2. Generate a config file as Table 1 below.
3. Choose the finished genome or upload a genbank file for the pathogen used in the screen
4. Upload the config .txt file
5. Press next
6. Analysis is performed with the following parameters (see Figure 2).
   a. Select TA for selected for mariner transposon mutant libraries.
   b. Select 30,000 for “library size”.
   c. Select yes for “perform repeat filtering”.
   d. Select 2 for “barcode mismatch”.
   e. Select 17 for “genomic sequence remaining of read”.
   f. Select bol for “barcode side”.
   g. Select eol for “transposon inverted repeat side”.
   h. Select 14 for “minimal sequence match”.
   i. Select 1(=forward) for “strand to align”.
   j. Select truncated.ptt for “3’ truncated genes for matching insertion site”.
   k. Select yes for “remove genomic position bias”.
   l. Select TMM for “normalization”.
   m. Select qCML for “paired analysis”.
   n. Select tagwise for “modeling of variance”.
   o. Select 10 for “amount of smoothing”.
   p. Select BH for “p-value adjustment method”.
   q. Select corrected for “p-value”.
   r. Select 20 for “minimal number of reads”.
   s. Select yes for “create ZIP archive”.

7. Press proceed

   Note: Analysis can take up to a few hours.

8. Unzip the created zip file and the file gene_alloutputmerged.tsv contains the data analysis for conditionally essential genes.

Table 1. Example for config file used for data analysis

link	barcode	transposon	sample type	library	sample format	compression
http://bamicsb.cmbi.ru.nl/tnseq/co2_Hinfluenza/s_1_export.txt	TCACG	ACAGGTT GGATGAT	target	lib1	export	none
http://bamicsb.cmbi.ru.nl/tnseq/co2_Hinfluenza/s_1_export.txt	GATGT	ACAGGTT GGATGAT	control	lib1	export	none
http://bamicsb.cmbi.ru.nl/tnseq/co2_Hinfluenza/s_1_export.txt	TAGGC	ACAGGTT GGATGAT	target	lib1	export	none
http://bamicsb.cmbi.ru.nl/tnseq/co2_Hinfluenza/s_2_export.txt	GACCA	ACAGGTT GGATGAT	control	lib1	export	none
http://bamicsb.cmbi.ru.nl/tnseq/co2_Hinfluenza/s_2_export.txt	GATGT	ACAGGTT GGATGAT	target	lib1	export	none
http://bamicsb.cmbi.ru.nl/tnseq/co2_Hinfluenza/s_2_export.txt	TCACG	ACAGGTT GGATGAT	control	lib1	export	none
http://bamicsb.cmbi.ru.nl/tnseq/co2_Hinfluenza/s_2_export.txt	GACCA	ACAGGTT GGATGAT	target	lib1	export	none
http://bamicsb.cmbi.ru.nl/tnseq/co2_Hinfluenza/s_2_export.txt	TAGGC	ACAGGTT GGATGAT	control	lib1	export	none

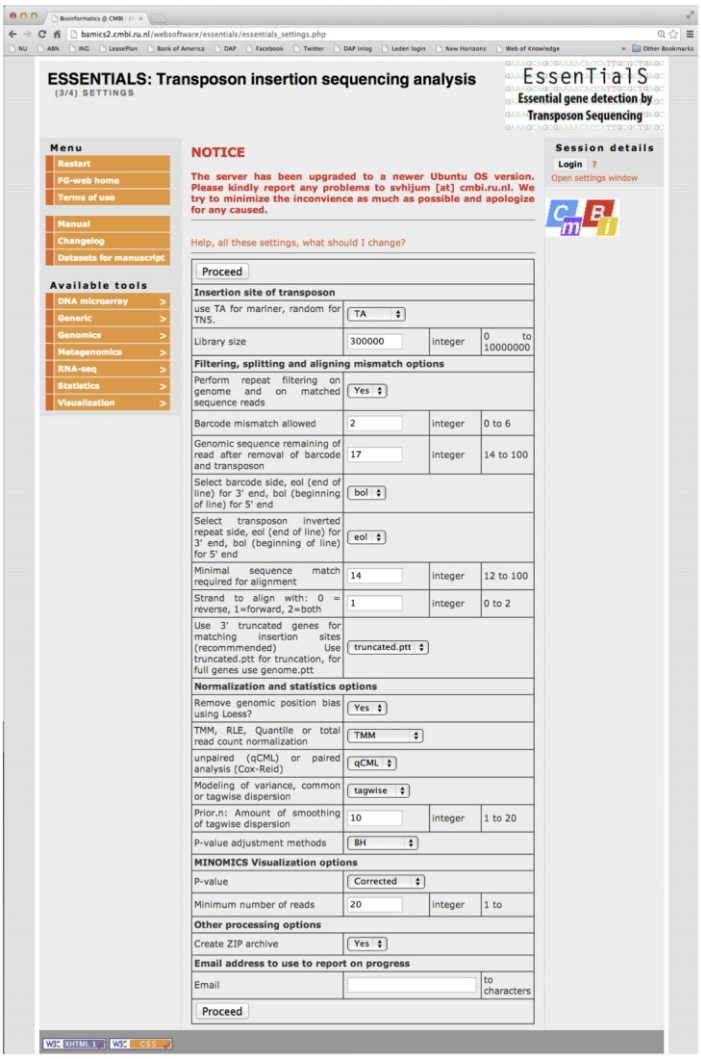

Figure 2. Screenshot of the analysis parameters on the ESSENTIALS website

Acknowledgments

This protocol is adapted from a previously published paper: Langereis *et al.* (2013).

References

1. Herriott, R. M., Meyer, E. M. and Vogt, M. (1970). Defined nongrowth media for stage II development of competence in *Haemophilus influenzae*. *J Bacteriol* 101(2): 517-524.
2. Illumina sequencing technology. http://res.illumina.com/documents/products/techspotlights/techspotlight_sequencing.pdf.

3. Langereis, J. D., Zomer, A., Stunnenberg, H. G., Burghout, P. and Hermans, P. W. (2013). Nontypeable *Haemophilus influenzae* carbonic anhydrase is important for environmental and intracellular survival. *J Bacteriol* 195(12): 2737-2746.
4. van Opijnen, T., Bodi, K. L. and Camilli, A. (2009). Tn-seq: high-throughput parallel sequencing for fitness and genetic interaction studies in microorganisms. *Nat Methods* 6(10): 767-772.
5. Zomer, A., Burghout, P., Bootsma, H. J., Hermans, P. W. and van Hijum, S. A. (2012). ESSENTIALS: software for rapid analysis of high throughput transposon insertion sequencing data. *PLoS One* 7(8): e43012.

Appendixes

Table 2. Primers used for tn-seq analysis*

Characteristics	sequence (5'-3')	Adapter
F primer with ATCACG barcode	TTCCCTACACGACGCTCTTCCGATCTATCACGNN	A
R primer with ATCACG barcode	P-CGTGATAGATCGGAAGAGCGTCGTGTAGGGAAAGAGT-P	
F primer with CGATGT barcode	TTCCCTACACGACGCTCTTCCGATCTCGATGTNN	B
R primer with CGATGT barcode	P-ACATCGAGATCGGAAGAGCGTCGTGTAGGGAAAGAGT-P	
F primer with TTAGGC barcode	TTCCCTACACGACGCTCTTCCGATCTTTAGGCNN	C
R primer with TTAGGC barcode	P-GCCTAAAGATCGGAAGAGCGTCGTGTAGGGAAAGAGT-P	
F primer with TGACCA barcode	TTCCCTACACGACGCTCTTCCGATCTTGACCANN	D
R primer with TGACCA barcode	P-TGGTCAAGATCGGAAGAGCGTCGTGTAGGGAAAGAGT-P	
F primer with ACAGTG barcode	TTCCCTACACGACGCTCTTCCGATCTACAGTGNN	E
R primer with ACAGTG barcode	P-CACTGTAGATCGGAAGAGCGTCGTGTAGGGAAAGAGT-P	
F primer with GCCAAT barcode	TTCCCTACACGACGCTCTTCCGATCTGCCAATNN	F
R primer with GCCAAT barcode	P-ATTGGCAGATCGGAAGAGCGTCGTGTAGGGAAAGAGT-P	
F primer with	TTCCCTACACGACGCTCTTCCGATCTCAGATCNN	G

CAGATC barcode		
R primer with CAGATC barcode	P-GATCTGAGATCGGAAGAGCGTCGTGTAGGGAAAGAGT-P	
F primer with ACTTGA barcode	TTCCCTACACGACGCTCTTCCGATCTACTTGANN	H
R primer with ACTTGA barcode	P-TCAAGTAGATCGGAAGAGCGTCGTGTAGGGAAAGAGT-P	
F primer with GATCAG barcode	TTCCCTACACGACGCTCTTCCGATCTGATCAGNN	I
R primer with GATCAG barcode	P-CTGATCAGATCGGAAGAGCGTCGTGTAGGGAAAGAGT-P	
F primer with TAGCTT barcode	TTCCCTACACGACGCTCTTCCGATCTTAGCTTNN	J
R primer with TAGCTT barcode	P-AAGCTAAGATCGGAAGAGCGTCGTGTAGGGAAAGAGT-P	
F primer with GGCTAC barcode	TTCCCTACACGACGCTCTTCCGATCTGGCTACNN	K
R primer with GGCTAC barcode	P-GTAGCCAGATCGGAAGAGCGTCGTGTAGGGAAAGAGT-P	
F primer with CTTGTA barcode	TTCCCTACACGACGCTCTTCCGATCTCTTGTANN	L
R primer with CTTGTA barcode	P-TACAAGAGATCGGAAGAGCGTCGTGTAGGGAAAGAGT-P	
Amplification primer 1	CAAGCAGAAGACGGCATACGAAGACCGGGGACTTATCATCCAACCTGT	
Amplification primer 2	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT	

*All primers were PAGE purified; P, phosphorylated; Barcodes are based on Illumina TruSeq Technology